

Title

Method of treating heparin-induced thrombocytopenia

This application claims priority of Provisional

5 Application Serial No. 60/108,432 filed November 13, 1998.

Field of the Invention

This invention relates to medical science particularly
the treatment of heparin-induced thrombocytopenia with
10 protein C.

Background of the Invention

Protein C is a vitamin K dependent serine protease and
naturally occurring anticoagulant that plays a role in the
15 regulation of hemostasis by inactivating Factors Va and
VIIIa in the coagulation cascade. Human protein C
circulates as a 2-chain zymogen, but functions at the
endothelial and platelet surface following conversion to
activated protein C (aPC) by limited proteolysis with
20 thrombin in complex with the cell surface membrane protein,
thrombomodulin.

"Express Mail - mailing label number EM564068313US

Date of Deposit 5/11/01

I hereby certify that this paper or fee is being deposited with the United State Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231..

QUEEN Thomas
Printed Name

Queen Thomas
Signature

In conjunction with other proteins, aPC functions as perhaps the most important down-regulator of blood coagulation resulting in protection against thrombosis. In addition to its anti-coagulation functions, aPC has anti-inflammatory effects through its inhibition of cytokine generation (e.g. TNF and IL-1) and also exerts profibrinolytic properties that facilitate clot lysis. Thus, the protein C enzyme system represents a major physiological mechanism of anti-coagulation, anti-inflammation, and fibrinolysis.

Heparin is a frequently used anti-coagulant that prolongs the clotting time of blood by preventing the formation of fibrin by its ability to catalyze anti-thrombin-III (AT-III). The heparin-AT-III complex inactivates thrombin and other proteases of the coagulation cascade.

Heparin is administered parenterally in vascular surgery and in the treatment of postoperative thrombosis and embolism. Approximately 1 to 30% (average 5%) of patients receiving heparin have an immunologic reaction resulting in heparin-induced thrombocytopenia (HIT) [Phillips, et al., *Annals of Pharmacotherapy*, 28: 43-45, 1994]. These adverse effects may develop into a syndrome known as heparin induced thrombocytopenia and thrombosis syndrome (HITTS). Patients with HITTS are at substantial risk for a debilitating or life-threatening venous or arterial thrombosis, such as lower limb swelling or ischemia, stroke, or myocardial infarction, with a reported combined mortality and major morbidity of 25% to 37% [Boshkov, et al., British Journal of Haematology, 84:322-328, 1993].

HIT is one of the most important drug-induced immune thrombocytopenic disorders that a physician must manage. Its importance is evident for several reasons, including, the high prevalence of heparin usage; the high frequency of thrombocytopenia; the lack of a better alternative anti-thrombotic agent; and the concomitant occurrence of thrombotic complications. It has been clearly demonstrated that HIT is caused by the activation and aggregation of platelets induced by heparin-specific antibodies. Activated platelets have potent procoagulation activity, and by this mechanism, fibrin thrombus (which appear macroscopically pale white, consequently the "white clot syndrome") may form [Arthur, et al., Pathology, 17:82-86, 1985].

The treatment of HIT includes the discontinuation of heparin and the administration of alternative anti-thrombotic therapy. Unfortunately, the patients are undergoing heparin therapy because they have thrombo-emboli or are at high risk of thromboembolism and discontinuing heparin leaves them without anti-coagulation. Agents such as low molecular weight heparins, the heparinoid Org 10172, hirudin, or warfarin, have been administered after cessation of heparin therapy [Ratnoff, et al., Disorders of Hemostasis, Chapter 8, W.B. Sanders Company, Philadelphia; Laposata et al., Arch Pathol Lab Med, 22:799-807, 1998]. However, such therapy is not effective in many patients because Org 10172 and low-molecular weight heparins can cross react with the heparin-specific antibodies of some patients with heparin induced thrombocytopenia. Furthermore, warfarin takes several days to take effect and has also been linked to venous limb gangrene when administered to patients with HIT [Warkentin

et al., Ann Intern Med., 127: 804-812, 1997]. Therefore, a need exists to develop an effective therapy for the treatment of HIT.

5 The present invention is the first to describe the treatment of HIT with protein C. Protein C, with its anticoagulant and profibrinolytic activities, is useful for the treatment of arterial and venous thrombosis, including the fibrin rich "white clot syndrome", that occur in HIT patients.

Summary of the Invention

10 The present invention provides a method of treating a patient suffering from heparin-induced thrombocytopenia (HIT) which comprises, administering to said patient a
15 pharmaceutically effective amount of protein C.

The present invention further provides a method of treating heparin-induced thrombocytopenia in a patient in need thereof, which comprises administering to said patient a pharmaceutically effective amount of activated protein C
20 such that an activated protein C plasma level of about 2 ng/ml to about 300 ng/ml is achieved.

Detailed Description of the Invention

25 For purposes of the present invention, as disclosed and claimed herein, the following terms are as defined below.

Protein C refers to a vitamin K dependent serine protease with anticoagulant, anti-inflammatory, and profibrinolytic properties which includes, but is not limited to, plasma derived and recombinant produced protein
30 C. Protein C includes and is preferably human protein C

although protein C may also include other species or derivatives having protein C proteolytic, amidolytic, esterolytic, and biological (anticoagulant, pro-fibrinolytic, and anti-inflammatory) activities. Examples
5 of protein C derivatives are described by Gerlitz, et al., U.S. Patent No. 5,453,373, and Foster, et al., U.S. Patent No. 5,516,650, the entire teachings of which are hereby included by reference.

10 Zymogen - an enzymatically inactive precursor of a proteolytic enzyme. Protein C zymogen, as used herein, refers to secreted, inactive forms, whether one chain or two chains, of protein C.

15 Activated protein C or aPC refers to protein C zymogen which has been converted by limited proteolysis to its activated form. aPC includes and is preferably human protein C although aPC may also include other species or derivatives having protein C proteolytic, amidolytic, esterolytic, and biological (anticoagulant or pro-fibrinolytic) activities. Examples of protein C derivatives
20 are noted above in the description of protein C.

HPC - human protein C zymogen.

r-hPC - recombinant human protein C zymogen.

25 r-aPC - recombinant human activated protein C produced by activating r-hPC *in vitro* or by direct secretion of the activated form of protein C from procaryotic cells, eukaryotic cells, and transgenic animals or plants, including, for example, secretion from human kidney 293 cells as a zymogen then purified and activated by techniques well known to the skilled artisan and demonstrated in Yan,
30 U.S. Patent No. 4,981,952, and Cottingham, WO97/20043, the

entire teachings of which are herein incorporated by reference.

Plasma derived activated protein C - activated protein C produced by activating plasma HPC as described in Eibl, U.S. Patent No. 5,478,558, the entire teaching of which is herein incorporated by reference.

Continuous infusion - continuing substantially uninterrupted the introduction of a solution into a vein for a specified period of time.

Bolus injection - the injection of a drug in a defined quantity (called a bolus) over a period of time up to about 120 minutes.

Suitable for administration - a lyophilized formulation or solution that is appropriate to be given as a therapeutic agent.

Unit dosage form - refers to physically discrete units suitable as unitary dosages for human subjects, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical excipient.

Pharmaceutically effective amount - represents an amount of a compound of the invention that is capable of inhibiting sepsis in humans. The particular dose of the compound administered according to this invention will, of course, be determined by the attending physician evaluating the particular circumstances surrounding the case.

The present invention provides for the treatment of heparin-induced thrombocytopenia (HIT) with activated protein C. Protein C, with its anticoagulant and

profibrinolytic activities, is useful for the treatment of arterial and venous thrombosis, including the fibrin rich "white clot syndrome", that occur in HIT patients.

5 The protein C administered according to this invention may be generated and/or isolated by any means known in the art or as described in U.S. Patent No. 4,981,952, and U.S. Patent No. 5,550,036, herein incorporated by reference. For example, protein C can be produced by secreting full-length, soluble protein C, or biologically active polypeptide
10 variants of protein C from a cell which comprises (a) constructing a vector comprising DNA encoding protein C; (b) transfecting the cell with the vector; and (c) culturing the cell so transfected in culture medium under conditions such that full length soluble protein C or biologically active
15 polypeptide variants of protein C, is secreted. Further, the cell is a eukaryotic cell, e.g. mammalian cell such as Syrian hamster AV12 cell, human embryonic 293 cell, or Baby Hamster Kidney cell.

20 The protein C used in the treatment of HIT can be formulated according to known methods to prepare pharmaceutically useful compositions. For example, a desired formulation would be one that is a stable lyophilized product of high purity comprising a bulking agent such as sucrose, a salt such as sodium chloride, a
25 buffer such as sodium citrate and protein C or aPC.

The protein C will be administered parenterally to ensure its delivery into the bloodstream in an effective form by injecting the appropriate dose as continuous infusion for about 1 hour to about 240 hours.

Those skilled in the art can readily optimize pharmaceutically effective dosages and administration regimens for therapeutic compositions comprising protein C, as determined by good medical practice and the clinical condition of the individual patient. Generally, the amount of protein C administered will be from about 5.0 $\mu\text{g/kg/hr}$ to about 250 $\mu\text{g/kg/hr}$. Preferably, the protein C used in the treatment of HIT is activated protein C. The amount of aPC administered will be from about 1.0 $\mu\text{g/kg/hr}$ to about 96 $\mu\text{g/kg/hr}$. More preferably the amount of aPC administered will be about 1.0 $\mu\text{g/kg/hr}$ to about 50 $\mu\text{g/kg/hr}$. While more preferably the amount of aPC administered will be about 1.0 $\mu\text{g/kg/hr}$ to about 35 $\mu\text{g/kg/hr}$. Even more preferably the amount of aPC administered will be about 5.0 $\mu\text{g/kg/hr}$ to about 30 $\mu\text{g/kg/hr}$. Yet even more preferably the amount of aPC administered will be about 15 $\mu\text{g/kg/hr}$ to 30 $\mu\text{g/kg/hr}$. Still even more preferably the amount of aPC administered will be about 20 $\mu\text{g/kg/hr}$ to 30 $\mu\text{g/kg/hr}$. The preferable amount of aPC administered will be about 24 $\mu\text{g/kg/hr}$. The most preferable amount of aPC administered will be about 48 $\mu\text{g/kg/hr}$. The appropriate dose of aPC administered will result in a reduction of the thrombotic complications associated with HIT.

The plasma ranges obtained from the amount of aPC administered will be about 2 ng/ml to about 300 ng/ml. The preferred plasma ranges are from about 2 ng/ml to 200 ng/ml. Most preferably, plasma ranges are from about 30 ng/ml to about 150 ng/ml and still more preferably about 100 ng/ml.

Alternatively, the aPC will be administered by injecting one third of the appropriate dose per hour as a

bolus injection followed by the remaining two thirds of the hourly dose as continuous infusion for one hour followed by continuous infusion of the appropriate dose for twenty-three hours which results in the appropriate dose administered over 24 hours. In addition, the bolus injection will be administered via an intravenous bag drip pump or syringe pump at 2 times the normal rate for 15 minutes followed by 1.5 times the normal rate for 45 minutes. The normal rate i.e. that rate which has been determined to administer the appropriate dose level of the therapeutic agent per time period, is then continued for up to 240 hours.

The use of protein C in the treatment of HIT as presented in the present invention will provide a needed therapy for a potentially serious and debilitating disorder. The use of protein C is efficacious and avoids the complications such as bleeding tendency, toxicity and general side effects of currently available anti-coagulant agents.

The following examples are provided merely to further illustrate the present invention. The scope of the invention shall not be construed as merely consisting of the following examples.

Preparation 1

Preparation of Human Protein C

Recombinant human protein C (r-hPC) was produced in Human Kidney 293 cells by techniques well known to the skilled artisan such as those set forth in Yan, U.S. Patent No. 4,981,952, the entire teaching of which is herein

incorporated by reference. The gene encoding human protein C is disclosed and claimed in Bang, et al., U.S. Patent No. 4,775,624, the entire teaching of which is incorporated herein by reference. The plasmid used to express human protein C in 293 cells was plasmid pLPC which is disclosed in Bang, et al., U.S. Patent No. 4,992,373, the entire teaching of which is incorporated herein by reference. The construction of plasmid pLPC is also described in European Patent Publication No. 0 445 939, and in Grinnell, et al., 1987, Bio/Technology 5:1189-1192, the teachings of which are also incorporated herein by reference. Briefly, the plasmid was transfected into 293 cells, then stable transformants were identified, subcultured and grown in serum-free media. After fermentation, cell-free medium was obtained by microfiltration.

The human protein C was separated from the culture fluid by an adaptation of the techniques of Yan, U.S. Patent No. 4,981,952. The clarified medium was made 4 mM in EDTA before it was absorbed to an anion exchange resin (Fast-Flow Q, Pharmacia). After washing with 4 column volumes of 20 mM Tris, 200 mM NaCl, pH 7.4 and 2 column volumes of 20 mM Tris, 150 mM NaCl, pH 7.4, the bound recombinant human protein C zymogen was eluted with 20 mM Tris, 150 mM NaCl, 10 mM CaCl₂, pH 7.4. The eluted protein was greater than 95% pure after elution as judged by SDS-polyacrylamide gel electrophoresis.

Further purification of the protein was accomplished by making the protein 3 M in NaCl followed by adsorption to a hydrophobic interaction resin (Toyopearl Phenyl 650 M, TosoHaas) equilibrated in 20 mM Tris, 3 M NaCl, 10 mM CaCl₂,

-11-

pH 7.4. After washing with 2 column volumes of equilibration buffer without CaCl_2 , the recombinant human protein C was eluted with 20 mM Tris, pH 7.4.

The eluted protein was prepared for activation by removal of residual calcium. The recombinant human protein C was passed over a metal affinity column (Chelex-100, Bio-Rad) to remove calcium and again bound to an anion exchanger (Fast Flow Q, Pharmacia). Both of these columns were arranged in series and equilibrated in 20 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.4. Following loading of the protein, the Chelex-100 column was washed with one column volume of the same buffer before disconnecting it from the series. The anion exchange column was washed with 3 column volumes of equilibration buffer before eluting the protein with 0.4 M NaCl, 20 mM Tris-acetate, pH 6.5. Protein concentrations of recombinant human protein C and recombinant activated protein C solutions were measured by UV 280 nm extinction $E^{0.1\%}=1.81$ or 1.85, respectively.

Preparation 2

Activation of Recombinant Human Protein C

Bovine thrombin was coupled to Activated CH-Sepharose 4B (Pharmacia) in the presence of 50 mM HEPES, pH 7.5 at 4°C. The coupling reaction was done on resin already packed into a column using approximately 5000 units thrombin/mL resin. The thrombin solution was circulated through the column for approximately 3 hours before adding 2-amino-ethanol (MEA) to a concentration of 0.6 mL/L of circulating solution. The MEA-containing solution was circulated for an additional 10-12 hours to assure complete blockage of the

unreacted amines on the resin. Following blocking, the thrombin-coupled resin was washed with 10 column volumes of 1 M NaCl, 20 mM Tris, pH 6.5 to remove all non-specifically bound protein, and was used in activation reactions after
5 equilibrating in activation buffer.

Purified r-hPC was made 5 mM in EDTA (to chelate any residual calcium) and diluted to a concentration of 2 mg/mL with 20 mM Tris, pH 7.4 or 20 mM Tris-acetate, pH 6.5. This material was passed through a thrombin column equilibrated
10 at 37°C with 50 mM NaCl and either 20 mM Tris pH 7.4 or 20 mM Tris-acetate pH 6.5. The flow rate was adjusted to allow for approximately 20 min. of contact time between the r-hPC and thrombin resin. The effluent was collected and immediately assayed for amidolytic activity. If the
15 material did not have a specific activity (amidolytic) comparable to an established standard of protein C, it was recycled over the thrombin column to activate the r-hPC to completion. This was followed by 1:1 dilution of the material with 20 mM buffer as above, with a pH of either 7.4
20 or 6.5 to keep the protein C at lower concentrations while it awaited the next processing step.

Removal of leached thrombin from the protein C material was accomplished by binding the protein C to an anion exchange resin (Fast Flow Q, Pharmacia) equilibrated in
25 activation buffer (either 20 mM Tris, pH 7.4 or 20 mM Tris-acetate, pH 6.5) with 150 mM NaCl. Thrombin does not interact with the anion exchange resin under these conditions, but passes through the column into the sample application effluent. Once the protein C is loaded onto the
30 column, a 2-6 column volume wash with 20 mM equilibration

buffer is done before eluting the bound protein C with a step elution using 0.4 M NaCl in either 5 mM Tris-acetate, pH 6.5 or 20 mM Tris, pH 7.4. Higher volume washes of the column facilitated more complete removal of the

5 dodecapeptide. The material eluted from this column was stored either in a frozen solution (-20°C) or as a lyophilized powder.

10 The anticoagulant activity of activated protein C was determined by measuring the prolongation of the clotting time in the activated partial thromboplastin time (APTT) clotting assay. A standard curve was prepared in dilution buffer (1 mg/mL radioimmunoassay grade bovine serum albumin [BSA], 20 mM Tris, pH 7.4, 150 mM NaCl, 0.02% NaN₃) ranging in protein C concentration from 125-1000 ng/mL, while

15 samples were prepared at several dilutions in this concentration range. To each sample cuvette, 50 µL of cold horse plasma and 50 µL of reconstituted activated partial thromboplastin time reagent (APTT Reagent, Sigma) were added and incubated at 37°C for 5 min. After incubation, 50 µL of

20 the appropriate samples or standards were added to each cuvette. Dilution buffer was used in place of sample or standard to determine basal clotting time. The timer of the fibrometer (CoA Screener Hemostasis Analyzer, American Labor) was started immediately after the addition of 50 µL

25 37°C 30 mM CaCl₂ to each sample or standard. Activated protein C concentration in samples are calculated from the linear regression equation of the standard curve. Clotting times reported here are the average of a minimum of three replicates, including standard curve samples.

The above descriptions enable one with appropriate skill in the art to prepare protein C for utilization in the treatment of heparin-induced thrombocytopenia.

5

Preparation 3

Formulation of Activated Protein C

09553859 051101
10 A stable lyophilized formulation of activated protein C was prepared by a process which comprises lyophilizing a solution comprising about 2.5 mg/mL activated protein C, about 15 mg/mL sucrose, about 20 mg/mL NaCl, and a sodium citrate buffer having a pH greater than 5.5 but less than 6.5. Additionally, the stable lyophilized formulation of activated protein C comprises lyophilizing a solution comprising about 5 mg/mL activated protein C, about 30 mg/mL
15 sucrose, about 38 mg/mL NaCl, and a citrate buffer having a pH greater than 5.5 but less than 6.5.

The ratio of protein C:salt:bulking agent (w:w:w) is an important factor in a formulation suitable for the freeze drying process. The ratio varies depending on the
20 concentration of protein C, salt selection and concentration and bulking agent selection and concentration. Particularly, a ratio of about 1 part activated protein C to about 7.6 parts salt to about 6 parts bulking agent is preferred.

25

A unit dosage formulation of activated protein C suitable for administration by continuous infusion was prepared by mixing activated protein C, NaCl, sucrose, and sodium citrate buffer. After mixing, 4 mL of the solution was transferred to a unit dosage receptacle and lyophilized.

The unit dosage receptacle containing about 5 mg to about 20 mg of activated protein C, suitable for administering a dosage of about 0.01 mg/kg/hr to about 0.05 mg/kg/hr to patients in need thereof, was sealed and stored until use.

5

Example 1

A placebo-controlled double blind trial of LY203638
Recombinant Human Activated Protein C (rhAPC) in patients
with Heparin Induced Thrombocytopenia (HIT)

10 Heparin-induced thrombocytopenia (HIT) occurs in 1% of
patients treated with heparin for 7 days and in 3% of
patients treated with heparin for 14 days. Venous, or less
frequently, arterial thrombosis occurs in 50 % of patients
with HIT despite the discontinuation of heparin. In
15 patients affected with (HIT), heparin leads to the release
of platelet factor 4 from platelets. Heparin in complex
with platelet factor 4 triggers the release of the HIT-IgG.
HIT-IgG in complex with heparin and platelet factor 4 bind
to the Fc receptors of platelets leading to their
20 activation.

Platelet activation leads to the formation of thrombin
by the release of further platelet factor 4. HIT-IgG
recognizes a complex of platelet factor 4 and endothelial
heparin sulfate leading to expression of tissue factor on
25 the endothelial surface and activation of the clotting
cascade. Platelet factor 4 itself leads to thrombin
formation by its ability to neutralize heparin.

The treatment approach to patients with heparin induced
thrombocytopenia (HIT) includes not only discontinuing
30 heparin, but also preventing the thrombotic sequelae of the

syndrome. Inhibitors of thrombin synthesis are the agents most likely to do this. Recombinant activated Protein C (r-aPC) is one such inhibitor of thrombin synthesis. r-aPC blocks the formation of thrombin by inactivating Factors Va and VIIa in the clotting cascade. Infusion of r-aPC results in a dose-dependent prolongation of the activated partial thromboplastin time (aPTT) demonstrating this effect.

This trial aims to show that the infusion of r-aPC results in a statistically significant reduction in the combined endpoint of mortality, new thrombosis, and limb amputations compared to placebo in patients with heparin-induced thrombocytopenia.

Inclusion criteria include patients with heparin-induced thrombocytopenia diagnosed by: 1) a platelet count by 50% from baseline or to less than 150,000 following 5 days or more of heparin; and 2) a positive test for heparin dependent IgG using the platelet ¹⁴C -serotonin release assay.

Patients meeting inclusion criteria for HIT and having no exclusion criteria, have their heparin stopped. In addition to starting warfarin therapy, patients receive either placebo or r-aPC for 96 hours. r-aPC is given in a dose of 48 µg/kg/hr, which has been shown in previous human trials to raise the aPTT to 2X baseline.

The primary endpoint of the study is a combined endpoint of 30 day incidence of mortality, limb amputation, and new thrombosis. The placebo event rate is assumed to be 50 %. The safety of r-aPC is compared to placebo with regards to the occurrence of clinically significant bleeds

- 17 -

as an additional primary endpoint. The secondary endpoint of the trial is time to platelet recovery.